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Effects of Silver Nanoparticles Synthesized from *Cordia myxa* and *Ceratophyllum demersum* Extracts on the Osmotic Fragility Test of Human Red Blood Cells *in vitro*.

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Abstract

Plant extracts are a rich source of active chemicals; they have lately been employed in environmentally friendly nanoparticle synthesis. In one step of environmentally friendly synthesis, it is crucial that the biomolecules present in plant extracts can transform metal ions into small particles. The study aimed to investigate the influence of silver nanoparticles (Ag NPs) synthesized from an aqueous extract of *Cordia myxa* and *Ceratophyllum demersum* on the osmotic fragility of human red blood cells (RBCs). Blood samples were treated with extracts of these two plants (0.125, 0.25 mg/ml, 0.357, and 0.75 mg/ml), and an osmotic fragility assay was done. The results showed a significant ($p < 0.05$) increase in RBC hemolysis in the presence of the extracts. Additionally, the results of the osmotic fragility assay revealed that the extracts of *C. myxa* and *C. demersum* have a hemolytic effect; these two plant extracts have similar effects on the fragility test of RBCs. In addition, the hemolytic impact of silver nanoparticles synthesized from *C. myxa* and *C. demersum* extracts is lower than that of the original extracts. These effects of the extracts and Ag NPs from these two plant extracts are linked to several pharmacological characteristics of these extracts and the chemical constituents of the nanoparticles.

Keywords: *Cordia myxa*, *Ceratophyllum demersum* plant extracts, silver nanoparticle, osmotic fragility, RBCs.

تأثير دقائق الفضة النانوية المصنعة من مستخلصات نباتي البمبر والشنبلان على كريات الدم الحمراء للإنسان خارج الجسم الحي

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الخلاصة:

تعتبر المستخلصات النباتية مصدر غني بالمركبات الكيميائية الفعالة والتي كثر استخدامها بالأونة الاخيرة لتصنيع الجسيمات النانوية الصديقة للبيئة. الخطوة الاولى في التصنيع الصديق للبيئة هو استخدام

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الجزيئات الحيوية التي من ضمنها المستخلصات النباتية القادرة على تحويل الايونات المعدنية الى جسيمات صغيرة جدا (نانوية). الهدف من هذه الدراسة هو للكشف عن تأثير جسيمات الفضة النانوية المصنعة من المستخلصات المائية لنباتي البمبر والشنبلان على المقاومة الاوزموزية لكريات الدم الحمر للانسان خارج الجسم الحي. تمت معاملة عينات الدم للمستخلصات المائية للنباتين وبثلاثة تراكيز لكل نبات وهي 0.125 و 0.25 و 0.357 و 0.75 ملغم بالمل، ثم قيس التأثير الاوزموزي لهذه المستخلصات على كريات الدم الحمر. اوضحت النتائج زيادة معنوية في تحلل كريات الدم الحمر بسبب وجود المستخلصات النباتية ايضا اوضحت النتائج اختبار الهشاشة الاوزموزية لمستخلصات البمبر و الشنبلان ان لها تأثيرا محلا وان كلا المستخلصين لهما نفس التأثير على كريات الدم الحمر. وان التأثير التحليلي لجسيمات الفضة النانوية المصنعة من تلك النباتات كان اقل من تأثير المستخلص النباتي الاصلي. ان هذه التأثيرات للمستخلصات النباتية وجسيمات الفضة النانوية المصنعة منها ترتبط ببعض الصفات الصيدلانية العلاجية للمكونات الكيميائية للمستخلصات وجسيمات الفضة النانوية المصنعة منها.

Introduction:

One of the most significant technological advancements in recent decades has been nanotechnology. In the field of biological applications, the processes involve the creation of nanoparticles derived from plant extracts, a simple, inexpensive, and environmentally beneficial technology [1].

Compared to other biological activities, plants are significantly more efficient at producing nanoparticles. To counteract microbial resistance, a variety of nanoparticle types have been employed in different contexts, and the synthesized silver nanoparticles (Ag NPs) exhibit activity against a variety of clinical bacterial species [2], also possess anticancer properties [3].

One of the largest classes of the family Boraginaceae is *Cordia myxa*, also known as "Bumber" in Iraq. There are 300 species of this family known to science, which are found in warmer regions such as Asia, Central and South America, and Africa [4]. Due to its potent compounds, the plant has a wide range of medical applications. This plant contains the following: gum, mucilage, alkaloids, phenolic acids, sterols, flavonoids, saponins, and terpenoids. The anti-inflammatory and analgesic properties of *C. myxa* were identified, as well as its antimicrobial, anti-ulcer, cardioprotective, hepatoprotective, hematological, and wound-healing effects. The inclusion of *C. myxa* mucilage powder significantly modifies the tablet's properties and increases its mucoadhesiveness. One can create a topical cream with *C. myxa*. [5].

The important and attractive marine macrophyte *Ceratophyllum demersum*, belonging to the Ceratophyllaceae family, has limited support in conventional medicine as an analgesic, antipyretic, anti-inflammatory, astringent, antiulcer, antidiabetic, anticancer, and so forth; it also has antimicrobial potential, antidiarrheal and wound-healing activity, and analgesic action. The phytochemicals found in the plant *C. demersum* extracts include flavonoids, alkaloids, glycosides, tannins, and cardiac glycosides, among others [6]. Red blood cells (RBCs) are the primary cells responsible for gas exchange between tissues in the body [7]. Osmotic fragility of erythrocytes is a test that reproduces the capacity of these cells to bear differences in the osmotic surroundings. The impairment is often related to a diversity of pathologies, malignant tumors, cardiovascular dysfunctions and hemolytic anemia [8]. Numerous medical laboratories do not routinely perform the osmotic fragility test (OFT), a manual and time-consuming examination that is traditionally used to aid in the identification of numerous erythrocyte diseases [9]. The osmotic fragility test of human red blood cells is an essential part of assessing the health of the erythrocyte membrane. Additionally, they have

demonstrated potential for characterizing RBC storage lesions, evaluating the quality of donor blood when testing blood-contacting medical equipment for in vitro hemolysis, and assessing cell-salvage suction devices for sublethal cell damage [10]. However, slight differences are observed in the interspecies alterations in RBC deformability at several blood osmolality levels [11]. Remarkably, osmotic fragility shows variation across different animal classes and closely correlates with their specific systems [12]. Injured or lacking proteins in the membrane of erythrocytes, the reason for the disorder, causing a reduction in the superficial part and size of erythrocytes. Authorities usually use the osmotic fragility test [13]. The increase in membrane stability enabled this and reduced RBC osmotic fragility, due to nanoparticles' ability to interact with amino acids involved in the polymerization reaction within the RBC membrane [14].

The study aims to produce silver nanoparticles from *C. myxa* and *C. demersum* plant extracts and study their effect on the membrane stability of human RBCs in vitro.

Materials and Methods

Plant collection and grinding

Cordia myxa and *Ceratophyllum demersum* leaves were collected from the gardens of the College of Science at the University of Baghdad, then dried in an oven at 40 °C. After drying, an electric blender was used to grind them into a fine powder for later usage. The resulting powder was stored in an airtight container [15].

Preparation of plant extract

C. myxa powder, about 50 g, was add to 250 milliliters of boiling double- distilled water (D.D.W.), and the beaker was kept in a shaker for 24 h. Then it was filtered using muslin and then using filter paper. On an oven set at 40° C, the supernatant was placed on petri dishes to dry. After the extract dried, it was collected and three concentrations (0.125, 0.25, 0.357, and 0.75 mg/mL) were prepared using D.D.W., then refrigerated at 4 °C for further use in the synthesis of Ag NPs [15]. All these steps were repeated for the second plant (*C. demersum*).

Silver nanoparticles (Ag NPs) preparation

In order to produce the silver nanoparticles, 5 milliliters of a 10 mM of AgNO₃ solution was mixed with approximately 10 milliliters of stock supernatant, whereas silver nitrate was not used in a control treatment. These solutions were stored at room temperature in the sun for a week. After a week, the solution which had turned brown due to the production of Ag NPs, was collected for further characterization following two centrifugations at 10,000 rpm for five minutes [16].

The unique characteristics of Ag NPs

1. Observable in ultraviolet

UV-visible spectroscopy (Shimadzu, Japan) was used for the characterization of Ag NPs. The absorbance was measured at 200-800 nm after 4 milliliters of D.D.W. and 1 milliliter of Ag NPs were combined at room temperature [17].

2. FT-IR assay

Using the technique described by Sweedan and Abdul Majeed [18], the FTIR-8400S (Shimadzu, Japan) was used to investigate significant alterations in Ag NPs' surface structure and bonding (to detect the production of Ag NPs).

Osmotic fragility determination

To determine osmotic fragility, the same volume of plant extract working solution (50 µl) was combined with 50 µl of heparinized volunteer blood diluted in 0.9% sodium chloride

(NaCl). The mixture was left to stand at room temperature for fifteen minutes. Here, the modified method of Dacie and Lewis [19] for osmotic fragility determination was used. Centrifuge tubes were filled with 2 ml aliquots of NaCl-containing solutions at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9%. 20 microliter aliquots of human blood, pre-incubated with the plant extract, were placed into the tubes, and the components were mixed carefully before standing at room temperature for 5 minutes. After another mixing, the resulting mixture was centrifuged at 3000 rpm for seven minutes. At 540 nm, the absorbance of the supernatant was measured against a 0.9% sodium chloride solution. The identical protocols were repeated for two plant extracts and the Ag NPs produced from them. For the control samples, the identical protocols were applied; instead of using plant extract, 10 μ l of freshly heparinized blood was combined with 10 μ l of 0.9% sodium chloride solution.

Statistical Analysis

To determine the impact of various elements on study parameters, the Statistical Analysis System program was utilized [20]. In this study, the means were significantly compared using the least significant difference (LSD) method (ANOVA/two-way).

Results and Discussion

Ag NPs' characterization through color change

As seen in Figure 1, Ag NPs form, as indicated by the color change of the combined solution from pale dark to extremely dark.

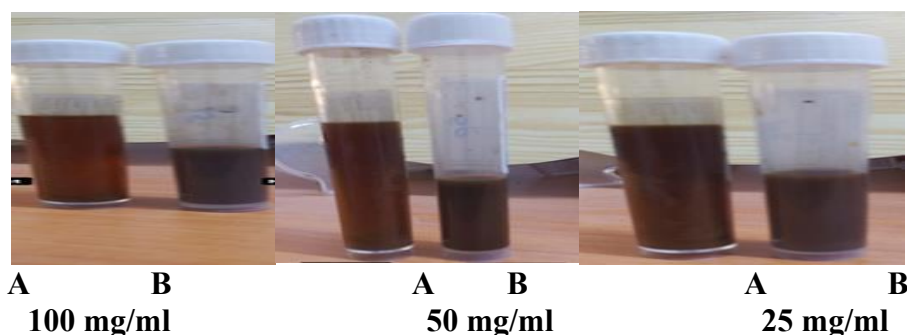


Figure1: Change in color of plant extracts at different concentrations subsequently the production of Ag NPs.

Spectrophotometer-based UV

Ag NPs' stated absorbance at 270 nm is displayed in Figure 2. A greater absorbance peak shows a decrease in the concentration of Ag NPs from *C. myxa* extract. While Ag NPs from *C. demersum* extract have a greater peak absorbance at 220 nm, as seen in Figure 3.

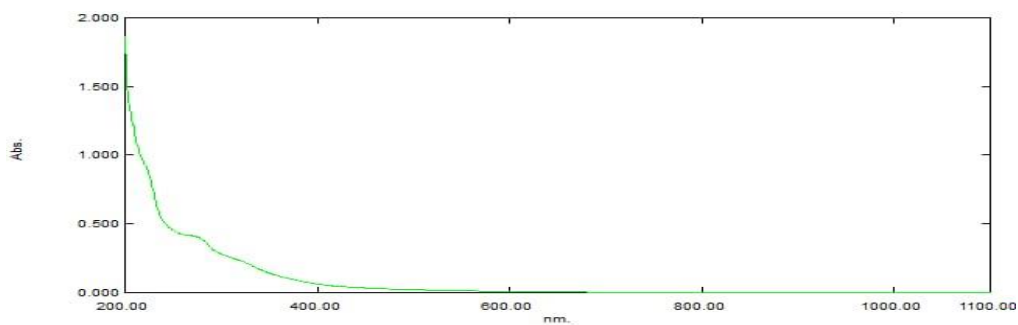


Figure 2: Using UV-Vis spectrophotometer to Ag NP from *C. myxa*.

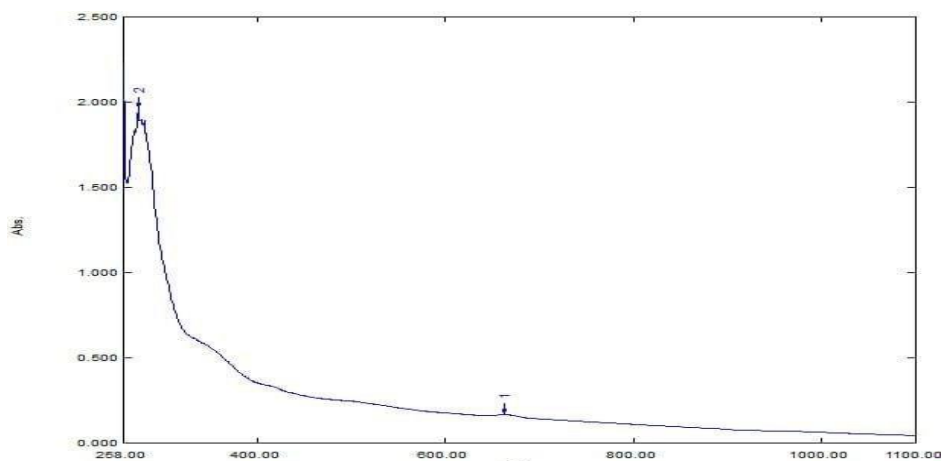


Figure 3: Using UV-Vis spectrophotometer to Ag NP from *C. demesum*.

Analyzing of FTIR

Findings indicated that solutions responsible for Ag NPs reduction and Ag ion bio-reduction were analyzed using an FTIR spectrum. Transmission bands found at the following wavelengths cm^{-1} [2] were indicative of the presence of a capping agent containing Ag NPs, as shown in Figures 4 and 5 for *C. myxa* and *C. demersum* extracts, respectively.

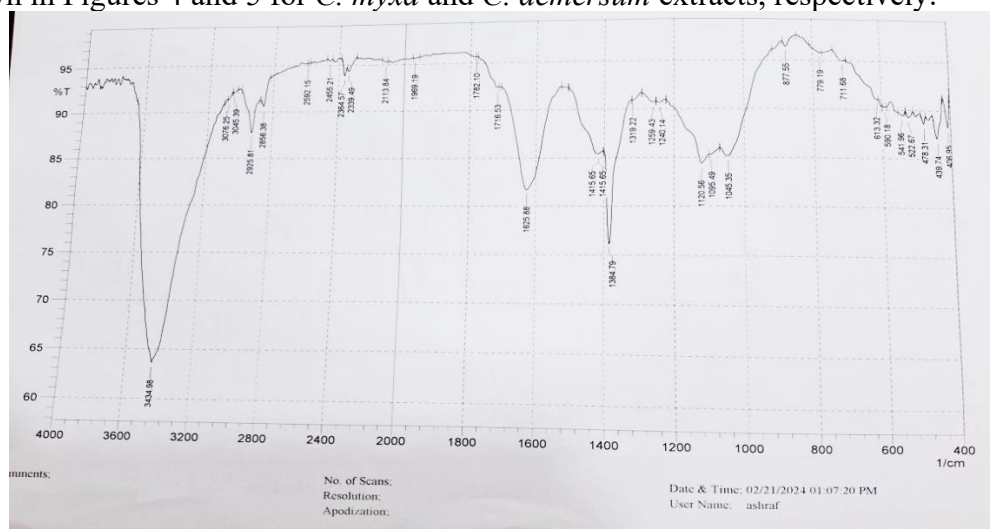


Figure 4: FTIR analysis of *C. myxa* Ag NPs.

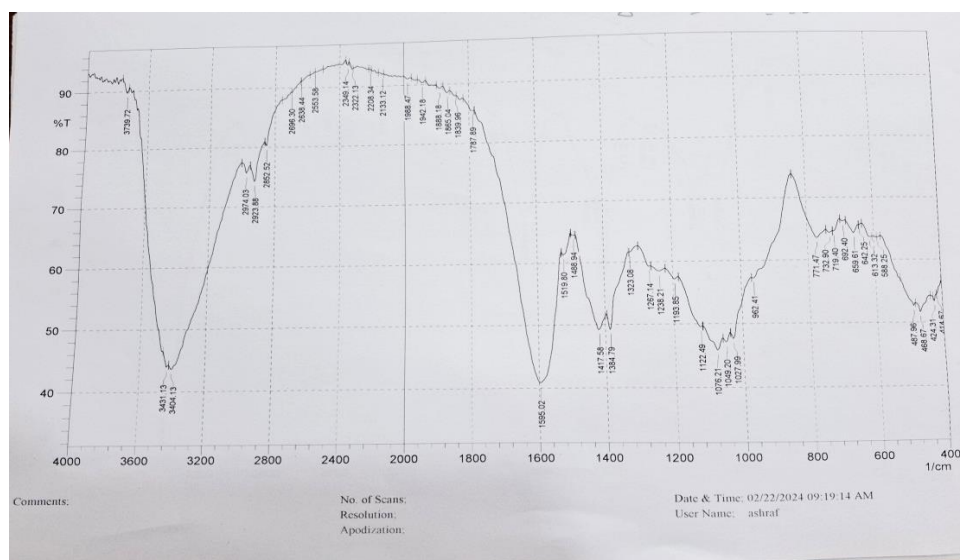


Figure 5: FTIR analysis of *C. demersum* Ag NPs.

The result of the study on the effect of *C. myxa* extract on the fragility test of blood samples showed significant differences ($P \leq 0.05$) (0.488, 0.502, and 0.569) of 0.125, 0.25, 0.357 and 0.75 mg/ml of *C. myxa* extract concentrations, respectively. In addition to significant differences in optical density (O. D.) at 540 nm, $P \leq 0.05$ LSD value (0.446, 0.492, 0.351, and 0.170) in NaCl concentration solutions (0.2, 0.3, 0.5, 0.6) respectively, while O. D. was non-significant LSD value (0.285, 0.119, 0.162, 0.169, and 0.157) in NaCl concentration solutions (0.1, 0.4, 0.7, 0.8, and 0.9), these results are summarized in Table 1.

Table 1: Effect of NaCl concentration and *C. myxa* extract on the fragility of RBC

NaCl concentration	<i>C. myxa</i> extract concentration mg/ml				LSD value
	0.125	0.25	0.375	0.75	
0.1	2.1725	2.0746	2.1572	2.0267	0.285 NS
0.2	2.0458	1.9102	2.4472	2.1219	0.446 *
0.3	2.1060	1.6063	2.3192	1.9383	0.492 *
0.4	.	0.1526	0.1855	0.2026	0.119 NS
0.5	0.373	0.7784	0.1834	0.1997	0.351 *
0.6	0.0471	0.2159	0.1818	0.2292	0.170 *
0.7	0.0379	0.1905	0.1979	0.1547	0.162 NS
0.8	0.0349	0.1642	0.1912	0.2037	0.169 NS
0.9	0.0522	0.1645	0.1934	0.1934	0.157 NS
LSD value	0.536 *	0.488 *	0.502 *	0.569 *	---
* ($P \leq 0.05$), NS: Non-Significant.					

The results showed significant differences ($P \leq 0.05$) in the LSD values of 0.533, 0.601, 0.549 and, 0.504 for four *C. demersum* extract concentrations (0.125, 0.25, 0.37 and, 0.75 mg/ml), and significant differences of O.D. 0.427 and 0.531 in 0.1 and 0.2 NaCl concentrations, but at other NaCl concentrations, the O.D. was non-significant: 0.408, 0.285, 0.417, 0.166, 0.154, 0.305, and 0.176 in NaCl concentrations (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9), respectively, as demonstrated in Table 2.

Table 2: Effect of NaCl concentration and *C. demersum* extract on the fragility of RBC

NaCl concentration	<i>C. demersum</i> extract concentration mg/ml				LSD value
	0.125	0.25	0.375	0.75	
0.1	2.2770	2.3546	2.4321	1.9630	0.427 *
0.2	2.1314	2.2443	2.2481	1.1586	0.531 *
0.3	2.3685	2.2874	1.9960	2.0569	0.408 NS
0.4	0.2362	0.2124	0.2316	0.4142	0.285 NS
0.5	0.6254	0.5952	0.2294	0.4010	0.417 NS
0.6	0.1829	0.2308	0.2400	0.2781	0.166 NS
0.7	0.1899	0.1655	0.2071	0.2528	0.154 NS
0.8	0.1851	0.1910	0.1258	0.4197	0.306 NS
0.9	0.1773	0.1891	0.1993	0.2547	0.176 NS
LSD value	0.533 *	0.601 *	0.549 *	0.504 *	---
* ($P \leq 0.05$), NS:Non-Significant.					

Some drugs have been reported to alter the physiology and structure of red blood cells [21]. The objective of the osmotic fragility test is to quantify red blood cells' resistance to hemolysis in response to plant extracts [22].

The results of the osmotic fragility assay used in the present study showed that *C. myxa* and *C. demersum* extracts have a hemolytic effect at certain concentrations, which may alter membrane integrity, and this effect is observed at almost physiological NaCl concentrations (Tables 1 and 3). Various methodologies have been employed to assess the consequences of drug-plasma membrane interactions [23]. Another technique that has been made accessible to determine the effects of natural materials on red blood cell (RBC) membranes is morphological examination [24].

The complex metric known as the critical hemolytic volume relies on both quantitative and qualitative parameters related to lipid and protein in the membrane. Thus, the crucial correlation that establishes osmotic fragility is the ratio of the red blood cell's internal osmotic contents to its essential hemolytic volume [25]. Additional research revealed that when rats were supplemented with low and high dosages of golden berry extract, the mean rate of hemolysis increased and osmotic fragility decreased [26].

Conversely, the result revealed significant variations $P \leq 0.05$ (0.589, 0.507, 0.617, and 0.563), of silver nanoparticles of *C. myxa* (0.125, 0.25, 0.37, and 0.75) mg/ml concentrations, respectively. Also, significant differences of O.D. (540 nm) $P \leq 0.05$ LSD value (0.406, and 0.512) in NaCl concentrations (0.2, and 0.5) respectively while non-significant differences LSD value (0.278, 0.364, 0.268, 0.276, 0.288, 0.286, and 0.294) of NaCl concentrations (0.1, 0.3, 0.4, 0.6, 0.7, 0.8, and 0.9) respectively, these results are summarized in Table 3.

Table 3: Effect of NaCl concentration and nanoparticles of *C. myxa* extract on the fragility of RBC

NaCl concentration	Nanoparticles from <i>C. myxa</i> extract concentration				LSD value
	0.125	0.25	0.375	0.75	
0.1	2.0224	2.0468	2.2832	2.2688	0.278 NS
0.2	1.8591	2.1312	2.2910	2.3180	0.406 *
0.3	1.8761	2.1751	2.2469	2.1514	0.364 NS
0.4	0.1324	0.3585	0.1528	0.1442	0.268 NS
0.5	0.5500	0.6321	0.5212	0.1588	0.512 *
0.6	0.3857	0.3841	0.1451	0.1419	0.276 NS
0.7	0.1406	0.3863	0.1755	0.3688	0.288 NS
0.8	0.2176	0.3845	0.1419	0.1393	0.286 NS
0.9	0.3592	0.3830	0.1428	0.3610	0.294 NS
LSD value	0.589 *	0.507 *	0.617 *	0.563 *	---
* ($P \leq 0.05$), NS: Non-Significant.					

On the other hand, the results revealed significant differences ($P \leq 0.05$) with LSD values of 0.548, 0.511, 0.507, and 0.407 for four concentrations of silver nanoparticles synthesized from *C. demersum* extract (0.125, 0.25, 0.37, and 0.75 mg/ml), and significant differences in O.D. ($P \leq 0.05$) were found in 0.3 NaCl concentration, and non-significant differences were found with LSD values (0.298, 0.304, 0.377, 0.286, 0.279, 0.296, 0.118, and 0.106) at 0.1, 0.2, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 NaCl concentrations, as shown in Table 4.

Table 4: Effect of NaCl concentration and nanoparticles from *C. demersum* extract on the fragility of RBCs.

NaCl concentration	Nanoparticles from <i>C. demersum</i> extract concentration				LSD value
	0.125	0.25	0.375	0.75	
0.1	2.2713	2.4050	2.2818	2.3161	0.298 NS
0.2	2.2728	2.1515	1.9713	1.1785	0.304 NS
0.3	2.0888	2.1222	2.1418	0.7633	0.537 *
0.4	0.1147	0.1826	0.1694	0.4501	0.377 NS
0.5	0.1657	0.0869	0.2372	0.2405	0.286 NS
0.6	0.1678	0.1405	0.2155	0.3891	0.279 NS
0.7	0.1252	0.2136	0.3784	0.1757	0.296 NS
0.8	0.1238	0.1204	0.1522	0.1948	0.118 NS
0.9	0.1256	0.1227	0.1563	0.2214	0.106 NS
LSD value	0.548 *	0.511 *	0.507 *	0.407 *	---
* ($P \leq 0.05$), NS: Non-Significant.					

According to the current study, the hemolytic effect of nanoparticles derived from *C. myxa* and *C. demersum* extracts is lower than that of the original extracts. Given that RBCs normally circulate for extended periods, attaching nanocarriers to their surface theoretically also accomplishes this purpose [27]. Apart from extending the lifespan of the carrier in the bloodstream, red blood cells provide a means for the glycocalyx to conceal associated cargo molecules and facilitate their distribution to blood-accessible targets such as RES sinuses, thrombi, immune cells, macrophages, and endothelial cells, to mention a few [28].

A number of laboratories have recently started investigating RBC-mediated nanocarrier transport. In this instance, when polymeric nanoparticles (NPs; 200 nm polystyrene spheres employed as model drug delivery vehicles) were absorbed by naive mouse RBCs, the resulting NP-RBC complexes injected into the mice's bloodstream behaved differently. The circulation period of RBC combined with nanoparticles was noticeably more extended than that of the unbound nanoparticles, as was to be predicted. Crucially, the removal of the carrier RBC was not accelerated by mixing nanoparticles with RBC at a ratio up to 50:1. Moreover, transport of reversibly linked nanoparticles to the pulmonary vasculature was made possible via RBC carriage [29].

The study disagrees with Anselmo *et al.*, [30], who stated that further cell line-reported toxicity and the mode of action associated with the increase in red cell fragility induced by Ag NPs have not yet been fully described [31]. According to earlier research, Ag NPs' small size and physicochemical characteristics, which promote rapid, direct membrane-enhanced permeability across cells, may be linked to the decrease in osmotic burst resistance observed after exposure [32].

Conclusion

According to the results of the osmotic fragility assay in the present study, the extracts of *C. myxa* and *C. demersum* have a hemolytic effect; both plant extracts have the same effect on RBC fragility. In addition, the hemolytic impact of silver nanoparticles synthesized from *C. myxa* and *C. demersum* extracts is lower than that of the original extracts, meaning an increase in the capacity of these cells (RBCs) to withstand differences in osmotic surroundings.

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Conflicts of Interest

The author declares that he has no competing interests.

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